problem over the years especially in the early days when the lesions in the attenuated strains were not really genetically defined.

Inbred mice exhibit varying levels of sensitivity to attenuated strains, and many of these attenuated strains have been, as EV76 is, pgm-minus. Some of them have other lesions, as well, and every strain of EV76 is different from every other strain of EV76, so it is also very hard to go over that part of the literature.

There are reports that CBA mice are more resistant to attenuated strains than C57 Black 6 mice.

Over the years, starting with Meyer basically, the mouse has been used to look at the passive protection model. Meyer used this to evaluate the response of human volunteers to potential plague vaccines, and this has already been discussed to a certain extent, but basically, the mouse is given I.V. the sera in question, and in about 30 minutes, the mouse is challenged over the right inguinal node with about 1,500 CFU of

Yersinia pestis.

There are some really strict standards to do the Mouse Protection Index correctly. Each time the test is done, the LD50 has to be repeated for that inoculum, and the LD50 should be no more than 12 CFU.

The organisms are monitored for 14 days, and the MPI is calculated. It's the percentage of mice dead during that period over the mean time to death. So, as this number gets larger as more mice die, in the meantime, the death is small, that number is very large, and that is not a good protective index.

But as you get more mice through monitoring, and the ones that do die take longer to die, the protective index gets lower, and that is an indication of protection.

There have been some recent demonstrations of vaccine efficacy in mice. One is the fusion protein of F1 and V, and you have heard a lot about those V antigens by now. This is actually a fusion of the two proteins expressed together, that was

originally made by Dave Heath, who is in the audience.

This, combined with alhydrogel, gives excellent protection against parenteral or aerosol challenge in Swiss-Webster outbred mice, and it protects, and this is very important against both F1-positive and F1-negative strains, because F1-negative strains do obtain virulence in the mouse model and in the nonhuman primate model.

Now, the study I just discussed used outbred mice. This is one of Diane's studies here that she already discussed, but just to go through briefly, they looked at inbred mouse strains. All of them responded well with good IgG1 titers.

They used alhydrogel as an adjuvant, so it was a Th2 type response, and this is an interesting point that I had not considered before, and that is that there is a problem with keeping male mice long term because of aggression, and this may affect the results of studies if male mice are used especially long term, but they did point that titers were maintained well in female mice.

DNA vaccines have been used in the mouse model. There were some early reports that a prime-boost approach worked well in inbred mice, but not in outbred mice. These immunizations were done IM.

More recent studies indicate that the gene gun might be a better approach as an immunization route and that combining it with a vector that targets expression to cytosol may be more successful, but to my knowledge, most of these later studies have been done with inbred strain mice, as well, so we really haven't resolved this issue.

The guinea pig is also the historical model of plague. It is quite sensitive to Yersinia pestis, but historically, there is reported to have been a seasonal resistance to infection, that is, during certain years, at certain times of the winter, the guinea pigs were not as sensitive to infection.

Some of it may be circumstantial. There was a belief among some early plague researchers that certain lots of guinea pigs were more

sensitive or resistant to plague in general, and that perhaps using a more inbred strain of guinea pig and a more consistent source would help their result.

It is known that F1 capsule is an important virulence factor in this model, although F1-negative strains are attenuated in the guinea pig, they are not in the mouse significantly, and they are not in nonhuman primates, so this is a difference between the guinea pig and other models.

For the most part, the disease resulting from subcutaneous infection is similar to that seen in mice.

The very early aerosol models are described in the guinea pig around the turn of the century, the previous century. Culture suspensions were described as being sprayed in the air, so these are probably multi-size particles, and for the most part, it did not really induce true pneumonic plague.

The necropsy showed cervical and laryngeal edema, cervical buboes, septicemia, and hemorrhage

of the intestinal wall. So, this is probably more of a pharyngeal plague being produced. In some cases, there was evidence of secondary lung infection, but not primary.

There were additional reports of intratracheal installation of organisms that could cause pneumonic plague although the percentage of animals that actually acquired true pneumonic disease from this was not clear.

Intranasal models were also discussed in the literature. These animals were anesthetized and about 10 percent of these organisms actually make it down to the lung, and it is interesting to note that some guinea pigs did transmit the infection to control cagemates during these studies, but it is not clear whether it was pneumonic transfer or other methods.

There were some nice studies done by Druett in 1956 showing that the particle size affected the course of disease in guinea pigs. Guinea pig respiratory tract apparently does not allow particles greater than 4 microns to reach the

lungs.

Particles less than 1 micron initiate a bronchopneumonia, very characteristic of a few in pneumonic plague, and the larger particles deposit in the upper airways, and that is where you start to find the cervical nodes and septicemia, but not any primary pneumonia.

The guinea pigs, this is a more modern study. This is one that Sue Welkos put out a few years ago. The aerosol LD50 or type strain Colorado 92 is similar in the guinea pig to that of mice, but unlike the mouse, F1-negative strains were attenuated in the guinea pig model, and what Sue found was that parenteral infection was protracted and often not dose related, which is not a good thing in an animal model.

In general, what has been found is that guinea pigs respond better to live attenuated vaccines than to subunit vaccines. You can enhance the response to subunit vaccines by giving them a very large antigenic mass, much larger than other experimental models or by addition of oil-based

adjuvants, which are not really considered to be ideal in today's environment.

Passive protection of guinea pigs has not been particularly successful over the years.

Again, this is some of Diane's work that she already discussed. The response to F1 is more variable than that seen in the mouse. The response to F1 was slower than that to V, which is the opposite of what you see in the mouse. Guinea pig sera can passively protect mice, and there was some protection of guinea pigs observed, but not the level of protection that we would see with the mouse.

There was some evidence of bubo development in immunized animals and very long-term infections here, not a good model of acute disease, and again the response to F1-negative strains is different than it is in other animal models.

Live attenuated vaccines were extensively explored in guinea pigs. Again, like the mouse that you would expect, the results depended on the nature of the attenuation, but there were some

cases where excellent protection was demonstrated.

Some vaccine strains--this is a very interesting paper by Meyer--showed that strains that were essentially avirulent in guinea pigs killed nonhuman primates. The exact title of the paper I think was Strains Harmless to Guinea Pigs and Highly Virulent in Primates, which is a scary sentence in terms of looking at the guinea pig model.

So, here are some statements made over the years about the guinea pig. "The guinea pig is not a suitable animal for testing plague antiserum."

"In experimental plague immunization, the reaction of the guinea pig has been unique. It is not quite like any other animal model."

Finally, "The response of guinea pigs did not offer any improvement over mice in evaluating the efficacy of plague vaccines."

So, these reasons, along with some others that I have mentioned, really have led many investigators away from the guinea pig as an animal model.

A model that has not been as extensively explored is that of the rat. Chen and Meyer, along with Williams and Cavanaugh, and some other researchers in the seventies, did do some research with various types of rats, different species, what they just call the "Sprague-Dawley laboratory rats," I am no exactly sure which one that is, and they found that the subq lethal dose was significantly higher than that of mice or guinea pigs, and that they found resistance to infection, not just in animals trapped in endemic areas, but also animals taken from areas which were not endemic and laboratory rats themselves, so this didn't appear to be acquired immunity.

As a side line, it is interesting to note that they found antibody and resistance to infection was transferred to the progeny of immunized animals.

Based on these results, they divided rats into three groups which they believed to be genetically different. The first are susceptible rats which die from a fairly small dose

consistently.

The second, partially resistant, that is, they survive a small dose. Some of them seroconverted and acquire immunity to a larger dose. Those which do not seroconvert remain susceptible to the higher dose.

What they call resistant, which survive, do not seroconvert, and they remain resistant to even higher doses. There is some evidence that this may indeed be genetic, and, in fact, they derive their strength from the Wistar rat, which they call "WR," and I am assuming it stands for Walter Reed since the researchers were from there. That was highly susceptible to plague regardless of the age of the rat, the sex of the rat, or the season of the year.

So, apparently the seasonal issue was one for rats, as well. Subcutaneous could be, you could use either subcutaneous or an intranasal challenge. They did point out, Williams and Cavanaugh, that intranasal was not as reliable as aerosol, that it led in the rat model to involve

larynx and the tonsils, but they believed it to be a more stringent test of vaccines than subcutaneous challenge.

It has been very rare to find documentation of direct comparisons between animals using the same strain, the same exposure conditions, et cetera, but this is one of them. This is Meyer, Quan, and Larson 1947 looking at intranasally induced pneumonic plague in mice, guinea pigs, and cotton rats.

They did find that they got primary pneumonia in all three models. The mice and the rats had a progressive disease that eventually led to death between 72 and 96 hours. Guinea pigs, however, were not visibly ill until they suddenly dropped dead between 72 and 96 hours, which also doesn't mimic the human condition.

The infection in the guinea pigs in this case was confined to the respiratory tract, whereas, it was more disseminated in the rat and mice.

A model that has not been excessively

explored, but which is interesting, are two species of the multimammate mouse. One of them is inherently more sensitive to Yersinia pestis than the other, M. coucha being more sensitive. There were laboratory colonies established of these models at one time.

It was put forth that perhaps this species might more closely mimic the susceptibility of the nonhuman primate to attenuated live vaccine strains than did the mouse or the guinea pig. This was based on one very small study looking at the strain where the guinea pig was not effective, and these monkeys were dying.

This model also reacted, it was very sensitive to this strain in the same way as a nonhuman primate, so they presented this as an alternative to the guinea pig in that it looked more like a nonhuman primate model in terms of attenuated strains.

It is thought or at least proposed that the difference between these two species of multimammate mouse is that the more resistant

species react to antigens of Yersinia pestis nonspecifically, so there may be an innate resistance there that protects the animal.

There have been Vole models used, also bred in the laboratory, and there has been a small amount of genetic work on this looking at the nature of resistance and there may be an association although it has not been definitively shown between phagocytic activity and resistance at challenge.

One thing that we haven't really discussed, but that should be kept in mind when choosing an animal model is that there is some host specificity of Yersinia pestis. As I have already mentioned several times, F1-negative strains are virulent in mice and in nonhuman primates, but significantly attenuated in the guinea pig.

Certain auxotrophs are also more attenuated in the guinea pig than in the mouse. These include aro mutants, purine auxotrophs, and asparagine auxotrophs, and the asparagine connection has been explored and this is actually

quite interesting. Lynn Boroughs did this.

Guinea pigs have an asparaginase in their sera, which degrades asparagine. So, in that animal model, there is no asparagine available for the asparagine auxotrophs, and the organism is unable to grow, and thus is attenuated. Mice do not have asparaginase. So, that is a very elegant study done I believe in 1971 with some major Brazilian strains of Yersinia pestis that were asparagine-negative.

There are some isolates also from the former Soviet Union, which are virulent for a number of models, but not for guinea pigs, and the reason for this has never really been explained. It is my understanding that these strains are not asparagine auxotrophs, so there has to be another explanation.

So, in conclusion, the mouse is the best established and accepted model in my opinion. The guinea pig has numerous drawbacks, some of which I have mentioned and some which have been described by other people.

There were other interesting models, but they haven't been as well developed, and they need further exploring.

Going back to Otten, it doesn't look like we have really gotten very far since 1936. "It appeared that the nature of the experimental animal was by far more essential to the results than the nature of the vaccine use." Some wise words from people in the past.

So, I will take any questions.

[Applause.]

DR. LYONS: Questions?

DR. : Pat, I just wanted to comment that there several different labs have recent experience with intranasal installation, and I know Rick Lyons has done some, we have been doing it, I think Sue Straley has done some--not yet? Okay. And Virginia Miller.

At least three different pestis strains have been used, several different strains of inbred mice, I don't know that anyone has used outbred. They are all finding that, you know, we get LD50s

on the order of 3- to 500 bugs.

DR. WORSHAM: That would be the general dose?

DR. : That is the delivered dose.

DR. WORSHAM: That is the dose that you deliver into the nares.

DR. : Delivering 50 microliters generally, I think. Generally, all of the lobes of the lungs are involved, the mice are bacteremic within 24 hours, and it seems like a pretty good model for pneumonic infection. I don't want people to go away with the impression that you can't get something that looks like pneumonic plague by doing intranasal.

DR. WORSHAM: You can get something that looks like pneumonic plague, but I have not seen the pathology that really describes the resulting disease processes and whether it is confined to primary pneumonic, or whether you also get cervical involvement. Have you seen that?

DR. : We haven't looked

carefully at the pathology with respect to cervical. I don't know if Rick has or not.

DR. LYONS: I think it is a lot like anything. The technique is critical to that, and we don't see it, but that doesn't mean, you know, giving something intranasally is not necessarily that easy, I mean to do it right. I think again, it is just learning the correct procedure and doing it right.

But I wanted to ask, I guess I am a little surprised even with inhalation that sometimes you don't see cervical, because clearly, most of those bugs are going either in the gut or up in the turbinates or someplace. I mean it is not a lung only, you know, there is a lot of bugs around.

So, has that been carefully looked at for inhalation, too, that there is no cervical nodes or anything, or is that just kind of assumed?

DR. WORSHAM: I think that has been looked at in some experiments. Many of these, a lot of this work is very old. It probably was not well quantitated, and I think it is probably a matter of

quantity. You see obvious cervical involvement or the animal dies, because if the primary pneumonic is strong enough, you may not have time to grow out cervical nodes.

DR. LYONS: Right, and I guess I would wonder if--and this is my own--if the cervical was the dominant mode, I would expect the kinetics to be a little different time to death. I mean pretty clearly, at least for subq versus intranasal or inhalation, the kinetics, the time to death is dramatically different, but I don't know about that. If you tried to infect the cervical node, what the kinetics would be. Do you have any clue?

DR. WORSHAM: I think that parallel experiments would be very nice. I think that that would be a nice study to do where you have control animal sets, strain sets, gross methods to the organism, and actually look at that kind of thing, I think that would be very interesting, but intuitively, it seems like there might be some differences between installing a rather large volume in the nares versus a small particle

aerosol, whether that is relevant, to a large degree, looking at vaccine efficacy, is another question altogether.

DR. : I am curious. Why do you think that the LD50 is so much higher for the aerosol? You are reporting about 4,000? It is about more a magnitude higher than what we see with intranasal.

DR. WORSHAM: I think that there are a lot of variables here that make it very difficult to answer that question. Is it the strain involved? Is it the method of growing the strain? Are some of the organisms damaged by the aerosol?

It could be the mouse strain is different. Like I said, it would be really nice to do some studies in parallel where we are looking to try to control some of these variables, because historically, that makes it very hard to look at this work.

DR. LYONS: Is that calculated dose, or is that actually--are the lungs removed?

DR. WORSHAM: Those are calculated dose.

DR. LYONS: That could explain the whole thing, it probably is.

DR. : I would just reiterate that for people that are doing these studies, they ought to do careful histopathology of the upper respiratory tract. It jogged my memory, and maybe somebody else here can remember from USAMRIID, but my recollection was that Kelly Davis had found, in the nalt antigen, very early--this is after aerosol challenge--

DR. WORSHAM: Mice?

DR. : My recollection was in mice, but I could be wrong. It was a long time ago.

DR. WORSHAM: You didn't publish it.

DR. : I know that, but it is something that people will ought to look at in the different models, because my recollection was that it occurred very early, which was a surprise to us.

DR. : I would just put out a cautionary note. When you use large volumes intranasally, we have actually measured this, and

we use 50 microliters, 90 to 95 percent of it ends up in the stomach, so you may be looking at part of the pathogenesis may be GI rather than a respiratory, so if you stay below 15 microliters, you tend to keep it out of the stomach, so it is something to think about as you do experiments.

DR. WORSHAM: And that is relevant because it was shown many years ago, I think, that you can infect animals orally with Yersinia pestis.

DR. LYONS: Thanks, Pat.

The final speaker for this is going to be Louise Pitt from USAMRIID talking on nonhuman primates as a model for pneumonic plague.

Nonhuman Primates as a Model for Pneumonic Plague

Dr. Louise Pitt

DR. PITT: Good afternoon.

Now for the very interesting and complicated topic on nonhuman primates and which model to use for pneumonic plague.

In order to understand and appreciate the models that are used today, I think it is very

important that we go back to the beginning because the work that was done right at the beginning has influenced all our decisionmaking to date.

It started off in the 19th century in Indochina where initially it was discovered by some Russian workers. They inoculated some nonhuman primates with the organism that is for this plague, three species of monkeys, and found that they were very susceptible.

It was only in 1933, though, when Taylor went back and identified these three species that were used - the three macaques, Macaca sinica, radiata, and Semnopithecus or the Presbytis entellus, which is also known as the Langur.

In 1898, then Yersin had what he called an "attenuated" strain. It actually killed rodents, but was termed attenuated. He, being a classical scientist, did what they did in those days, he took some macaques and himself, and he inoculated both. Both himself and the macaques got fairly ill, but survived, and his conclusion was that the susceptibility of the macaque was similar to man.

In 1899, some German scientists then put Yersinia pestis into the Langur and compared it to the Macaca radiata, saying that they were similar in susceptibility.

Again, in 1904, a nonpathogenic strain for guinea pigs was found to be virulent, in this case, in Cercopithecus aethiops, which is the Grivet, which is also the African Green monkey or the Verbit, very similar.

1907 was the first time when a cynomolgus macaque, the philippensis cynomolgus macaque was used, and it was determined that the susceptibility of the cynomolgus macaque lay somewhere between the Langur and the Macaca radiata.

Again in 1912, the cynomolgus macaque was used and shown to be much more susceptible than guinea pig.

Now, in order to put all this historical susceptibilities into perspective, we need to remember that all of these relative susceptibilities of these nonhuman primates was based on inoculation of the skin.

They all concluded that the macaque showed individual variations. This, of course, is very old studies, unknown where the macaque came from. Some were caught from the wild, the majority were actually caught from the wild. Studies were done under fairly primitive conditions. There was lack of technique standardization, cultures varied, very little information as to strain information. So, all this data needs to be taken in that perspective.

However, there was always one conclusion that was agreed upon across all the literature, that regardless of susceptibility, once the animals became ill, the disease was very similar across the different nonhuman primates and was also similar to what was seen in humans.

This is just a summary of the table from Meyer in 1954, where he showed a single--this is 195/P, a virulent strain of Yersinia pestis. As you can see, many experiments with Macaca mulatta, also subq route, but you get survivors from 2 logs to 9 logs, and in cynomolgus macaque, 10^6 and 10^7

given subq, you still get survivors.

The conclusion was always that the macaque was susceptible to plague Yersinia pestis, but that there was individual variability, considerable individual variability. A group of animals brought in from a single site could have an incredibly different susceptibility to Y. pestis.

Now, moving on to pneumonic plague, the initial study by Ehrenkrantz and Mayer in 1955 looked at Macaca mulatta, the Rhesus macaque. This was actually intratracheal, not an aerosol exposure, and came to the conclusion that about 100 CFU of this 195/P strain killed more than 50 percent of the animals. So, quite a difference from the skin inoculation route.

Speck and Wolochow, in 1957, did some aerosol work. This was small particle aerosol using the Macaca mulatta again, the Rhesus macaque, and concluded that their LD50 was around 2 x 10^4 of the 139L, a virulent Y. pestis strain.

On looking at this again several times, it appeared that there was a vaccine study going on at

the same time and any animal that died was actually included in this LD50 estimation, including animals that had been partially immunized with a vaccine, so any animal on this trial was included in the LD50 study, and this could very well be why the LD50 is so high in these studies.

Moving on now to the early 1990s, at USAMRIID, when we set about developing the nonhuman primate model for pneumonic plague, we did extensive literature research, had extensive discussions, and the species that was chosen was the Cercopithecus aethiops, which is known as Chlorocebus aethiops or the African Green monkey, and as I said, this was based on a very extensive literature review, because throughout the literature, this model was the one that was consistently consistent. That was the message across all the studies, that it was a very, very reliable consistent model.

We realized based on susceptibility that it was susceptible, probably more susceptible than the macaque, but we chose it because we were

looking for a stringent model, and then based on our literature survey and then the work that I am going to show you now, the course of disease in this animal model is very similar to vaccine in humans, and death is due to primary pneumonia.

This is the analysis of the LD50 curves for the African Green monkeys, the strain of Y. pestis that was used in Colorado 92. This is the standard strain that we have used at USAMRIID for all our challenges, whether they be for vaccine efficacy or therapeutic.

We have also used the F1-negative isogenic strain of Colorado 92, which is called C12, and the LD50 is around 343 CFU, and that is an inhaled or presented dose, so this is a very susceptible organism.

The LD99 based on this curve is around 50 LD50. We did do an LD50 study with the F1-negative strain and got an LD50 of 800 CFU, very similar.

Based on a study that was done not too long ago, this is the clinical pathway after an animal has been exposed. This is a natural history

study that was done in conjunction with a therapeutic study, but it shows the model very nicely.

The animals are exposed at time zero. They have telemetry devices in them, so we monitor their temperature all the time, and you can see they are perfectly normal 24, 48 hours. Around 72 hours, the temperature increases, they have a fever until they succumb and die. Usually, we euthanize the animals whenever possible when they become moribund.

This is an example of an animal that received 57 LD50. This temperature curve is very consistent, this model is very consistent. At the time when they are getting a fever, there are no clinical signs, but within 12 to 18 hours, they do start to show clinical signs. At the time that they show clinical signs, they are bacteremic, and then death occurs fairly rapidly after that.

This is the heart rate curve showing a very similar pattern to the fever curve.

This is showing you the respiratory rate

that is fairly steady until you get towards the last 12 hours or so prior to death where you get a massive increase in the respiratory rate.

This is the pattern of the white blood cells showing around 72 hours you get that inversion of the lymphocytes, granulocytes with the monocytes staying fairly constant.

These are radiographs of the same animal. This was taken pre-exposure. This radiograph was taken at 83 hours, at a time when the animals were showing clinical signs. You can see there is some infiltrates in the lung at this time, and at this time, the animal is bacteremic.

The last radiograph was taken when the animal was euthanized at 111.5 hours post-exposure.

This is just another view of those radiographs, before, during. The very rapid time course of this disease.

More recently, after many discussions with collaborators, et cetera, we went ahead and we developed the cynomolgus macaque as a comparison model. We did an LD50 based on the staircase

method, ranging in the 10^4 to 10^2 CFU range, and the LD50 came out to 400 CFU Colorado 92.

It turns out that the cynomolgus macaques are just as susceptible as the African Green monkey based on the data and the consistency of the experimental conditions. They die within the same time range, 4 to 5 days.

The onset of fever in animals receiving a lethal dose is similar, around 72 hours post-challenge, and the animals are normally moribund within 48 hours post-challenge.

Looking at the clinical signs between both African Green monkey and the cynomolgus macaque, no real difference whatsoever. Fever is the initial symptom. There is an increased respiratory rate. These animals then breathe extremely rapidly with labored breathing and rales.

They are usually euthanized when they are moribund, and at the time of euthanasia, pink froth just pours out of their mouth and nose.

This is just some of the comparative pathology that is being collected to date. We

don't have as many macaques as we do African Greens at this point.

This table just compares the control African Greens to vaccinates that have died, and smaller numbers of untreated macaques, and we have, in this table, only two vaccinated macaques that have died.

In the African Green, the symptoms are very similar in the vaccinate versus untreated, and the untreated African Green and the untreated macaques have very similar pathology.

Now for some pathology. I am not a pathologist, but I have to show you some pathology.

This top one is the normal lung. This is a vaccinate, exposed lung from an animal that is a vaccinate that died, and this is the control lung. This is actually from a cynomolgus macaque. If the slide was from an African Green monkey, it would look exactly the same.

Here, you can see there are neutrophilic infiltrates in the lung. This is pathology of the spleen, the red is spleen. The blue in here is

bacteria, and again you can see that there are neutrophilic infiltrates.

Here, there is liver, and this is a fibrin thrombi that is covered with bacteria.

So, in comparing the African Green monkey and the cynomolgus macaque to date, based on clinical signs, the disease progression, and the pathology, as well as susceptibility in terms of an LD50, they are very similar, very similar in susceptibility, pathology, and disease progression, and both are very similar to what is known about the human disease.

So, now moving on to vaccine efficacy, first of all, in the African Green monkey, the initial study that was done at USAMRIID back in the early 1990s was looking at the plague USP, the licensed vaccine. You will notice the Cutter vaccine.

We had 12 vaccinates and 6 controls. They were given the licensed schedule, at day zero, 28, and 91, and then the animals were challenged about 7 weeks later. The challenge dose was around 118

LD50, and there were no survivors, and there was no difference between mean time STAT.

At that time, we measured antibody responses to F1, and I did not bring the data, but at time of challenge, the F1, anti-F1 titers were very low. With this vaccine, the IgG titer would go up after every boost, but then come down fairly rapidly.

Now, moving on to the more modern times, efficacy of the candidate recombinant F1-V fusion protein vaccine that was developed at USAMRIID. The study design, and this is a competent study design because I am going to show you several studies.

Basically, the vaccination route was always intramuscular. The F1-V fusion protein was always combined with alhydrogel. There were either 2 or 3 doses given. The challenge was always 6 weeks after the last dose, and, of course, the challenge was always the small particle aerosol, and we used either the Colorado 92 or the F1-negative strain.

Some results. The first study, the animals received 2 doses of 30 micrograms of the F1-V at zero and 28. The challenge strain in this study was V12 with an average of 55 LD50 as the challenge dose, and 2 out of 4 survived, whereas, the one control succumbed to pneumonic plague.

In the next study, the next group of animals received 2 doses of the 30 at zero and 28, and then at 3 months, received a 300 microgram dose of F1-V to see if we could boost the survival. They were again challenged with the V12 strain with a higher average LD50 of 259, and 3 out of 4 animals survived while the control succumbed.

In the next study, animals received 150 micrograms of F1-V 3 times, at zero, 28, and 56. The challenge dose in this study was much higher, at around 600 LD50, and 4 out of 10 survived while the control died.

The next study, again F1-V, 150 micrograms, 3 doses, the same schedule. This time they were challenged with Colorado 92 with the average dose of 166 LD50, with 2 out of 10

surviving.

The final study, again, the exact same study design, 150 3 times. Challenged with Colorado 92 with a lower challenge, but zero out of 10 survived in that trial.

This is the immune response data from the last study that was shown, showing that the animals got antibody to F1, a fairly consistent response. All the animals responded to F1 and had IgG. The antibodies to V in the African Green, it is a very varied response, and this is pretty much typical of all studies that have been done, that the response to V antigen in the African Green is a very individual and varied response.

Moving on to vaccine efficacy now in the cynomolgus macaque. First of all, the recombinant F1-V fusion, the USAMRIID candidate. The study design, basically, very much the same as the African Green monkey study with 3 doses given intramuscularly, challenged 6 weeks after the last dose.

This is the immune response data for both

F1 and V, and in the cynomolgus macaque, the V response is much more consistent.

Results. Three trials to date. The first, 150 micrograms given 3 times, zero, 28, and 56 days, just like with the African Green. The challenge strain Colorado 92. The average challenge 72 LD50 with 80 percent survival, 8 out of 10.

The second study. Again, exactly the same study design. In this, a very low LD50 challenge was given. All the animals survived and both controls died.

Again, the third trial with 160 LD50 average, challenged, 8 out of 10 survived.

Now moving on to the other recombinant F1 and recombinant V protein vaccine. This is the vaccine developed at DSTL in the UK, and this was a collaborative study between the DSTL and USAMRIID with funding from the Joint Vaccine Acquisition Program here at DoD.

The study design, the vaccine antigens are the recombinant F1 and the recombinant V protein

that are combined with 20 percent volume to volume alhydrogel. The vaccination route is intramuscular. Two doses were given, and as standard with all the vaccine trials, the challenge was 6 weeks after the last dose.

This is the immune response data for both the F1 and the V antigen, again showing the consistency of the response of the cynomolgus macaques, both F1 and V.

That first one was for the 40 microgram. I forgot to mention there were 2 doses of this vaccine given. The one group got 40 micrograms of F1 and 40 micrograms of V, and in the second group, it was 80 micrograms of F1 and 80 micrograms of V, and this is the immune response to the second group that got 80 plus 80.

The results. The group that got 40 plus 40, the schedule was zero and 21 days, the 2-dose schedule. The average challenge for this study was 126 LD50. We did lose one animal prior to challenge to an unrelated event, and 8 out of 9 animals survived with the 40 plus 40 dose.

In the 80 microgram plus 80 microgram, 10 out of 10 survived, and the 2 controls died.

So, to summarize to date, it appears based on the disease process and the clinical signs and what is known of the human disease, both African Green monkey and the cynomolgus macaques are appropriate models for pneumonic plague, and recombinant F1 and V-based vaccines do provide protection against the lethal aerosol challenge, and as I said previously when we started, we chose the African Green because we felt that they would be a more stringent model, and I think the results to date have proven that they really are a very stringent model in terms of vaccine efficacy and a very high bar.

Time will tell which is the most appropriate model.

Thank you.

[Applause.]

DR. LYONS: We have time for questions.

Louise, I just have one quick question. I don't know if you have any information, but is the

poor immune response, do you know if that is just to V antigen, or is that characteristic of green monkeys for any antigen that you are aware of?

DR. PITT: The F1 was characteristic. It is pretty much the same. We are in the process of looking at other antigens in African Greens to see what that looks like, but in terms of this, in all the literature, there isn't anything to point the way, no.

DR. : This might be a very naive question. Given the wide variation of LD50, you see different study, even within sometimes the same group, and different challenge and strain. From previous, other presentations, people use index.

Is that possible to use something like that as a more quantitative or also included variable how long the animal will die instead of just LD50?

DR. PITT: In our hands, the LD50 is very low and very consistent both for the African Green and the cynomolgus, and that is with an aerosol model of pneumonic plague.

I think the variation in the species and susceptibility comes when you look at the skin inoculation, and I think you might see, if you do bubonic studies, that there might be quite different susceptibilities between these two species in terms of an LD50.

DR. BURNS: Drusilla Burns, CBER.

Louise, in your vaccination studies, your African Green monkeys weren't protected very well with the fusion protein. Do you think that was simply because of the very varied response that they had to the V antigen? Did animals that died have very low response to V, or was there no correlation?

DR. PITT: In terms of the small number of animals that we got to date, and the ELISA data, there doesn't appear to be any correlation other than some animals appeared to respond to V as if they have seen V before, and others appear to be more naive to V, and I think there needs to be a lot more work done on that subject to understand exactly what is going on.

I didn't have time to put up a lot of detail on different experiments, but if you look at the studies of the animals that survived in the African Green versus those that died, there is a trend towards the lower challenge dose, the more survive. So, that would lead us to that this is a much higher bar to reach in terms of the model.

DR. BURNS: Do you have any idea where humans are on this scale?

DR. PITT: I would not like to comment.

DR. : Have you established any sort of a target LD50 in selecting the LD50s that you use in the sense that what sort of level of exposure might be expected during a bioterrorism attack, for example, what level of protection do you need?

DR. PITT: That's a good question. There could be many answers, because it would depend on the scenario in which you would be exposed. Given that the LD50 is so low in nonhuman primates, and assuming that it is also low in humans, you wouldn't have to be exposed to very much for it to

be a lethal dose.

MS. SCOTT: Leah Scott, DSTL.

I was just wondering whether some of the variability issues that you saw with the African Greens might be explained in part by some of their sourcing issues and their rather different natural history that they would have been exposed to perhaps in early life.

Perhaps as a follow-on to that, would you see that as a potential problem area when one considers a requirement to do key studies to GLP in view of like breeding programs?

DR. PITT: Well, the African Green monkeys, actually, I think why such a consistent model is because all the animals we have ever received have come from the Island of St. Kitt, which is basically a closed colony, has been for over 300 years, so the source of the animals has actually been exactly the same.

MS. SCOTT: But they would have been exposed to a much wider range of natural stimuli presumably than most captive bred animals.

DR. PITT: Possibly.

MS. SCOTT: As I was saying to you earlier on, we are proposing to complicate the issue yet further by proposing, as we are trying to do in the UK, to look at the common marmoset as a potential model in these areas, and we have already characterized the species, so it would be very interesting to put all of these results together and compare.

DR. PITT: It certainly would, but I would also add that cynomolgus macaques are not captive bred. They are also brought in from outside sources, so they have also been exposed to external stimuli.

DR. MEYSICK: Karen Meysick FDA.

I was wondering if you would comment on the differences between cynos and the African Greens in terms of just their background. My understanding was that African Greens, I think are more susceptible or resistant actually to SIV.

DR. PITT: Right, they don't die from SIV, correct. They can be infected, but they don't

actually develop the disease.

DR. MEYSICK: As I related to, it is something to do with CD4s, I think, CD4 cells.

DR. PITT: Right. The TB ratio in African Greens is reversed compared to macaque, but on that subject, I think that is a very important discussion point when you are talking about animal models, how do we actually know about the animals themselves, let alone adding in organisms to make them sick.

DR. : I should know this, but were the serologies done by the same assay or the same group between the F1-V fusion and the F1 plus V?

DR. PITT: No, they were not.

DR. : It would be useful to do that.

DR. PITT: I think it would be a great exercise.

DR. : Because clearly, differences in the level of antibody, and that would be important to see if there are functional

differences.

The other point is in all of these studies, that somebody needs to consider what, if anything, is F1 doing.

DR. PITT: Yes.

DR. FERRIERI: Pat Ferrieri, University of Minnesota.

I have seen LD50s in the literature cited as 3,000 for humans. Do you have any notion where that would have been derived from, any data that you have seen anywhere?

DR. PITT: No, I think that is based on assumptions of what people know from the Manchurian outbreak, from information that has been received from different documents, but I have no idea where that number comes from.

DR. FERRIERI: Another quick question. I also have been concerned about the genetic lineages of these two types of nonhuman primates, and do you have multiple sources from which you obtain your animals, breeding facilities are different or not?

DR. PITT: As I said, the African Green

monkey, we have been very consistent with the source of the African Green monkey. The cynomolgus macaque has been a little different because they have been come in from the Seychelles, they have come in from the Philippines. They have come in probably from China, too, and India, I believe. So, there could certainly be differences in the cynomolgus macaque that we are not aware of, but the African Green source has been consistent since we started working on them.

DR. LYONS: Is the V antigen in the fusion, is that functional? Like if you put the fusion protein on cells, do you get the IL-10 response and that sort of thing, do you know?

DR. PITT: That is not my area of expertise. I believe it is functional.

DR. LYONS: I am just curious because it looked like on your slide, that the 30, you know, and this was small animals and everything, that the 30 tended to work better than the 150 dose, at least for CL12, and the question would be could you go up high enough with proteins, so that you now

you do bring out this local immunosuppressant phenotype, and actually by giving too much protein, you decrease your immune response.

I find that hard to believe.

DR. PITT: We have had those discussions.

[Recess.]

Session 4: New Data on Aspects of Plague Vaccine Development

Dr. Luther Lindler, Moderator

DR. MEYSICK: I think we will start the last session for today, which is new data on aspects of plague vaccine development, and the moderator for this session is Dr. Luther Lindler from the Department of Homeland Security.

DR. LINDLER: Thanks. This session is on new data on aspects of plague vaccine development, and the first speaker is going to be Sue Straley speaking to us about how does antibody against LcrV protect against plague.

How Does Antibody Against LcrV Protect Against Plague?

Dr. Susan Straley

DR. STRALEY: We are trying to discriminate among the various ways that anti-V could possibly protect against plague, and I would like to tell you about one of our stories.

Our model is Yersinia pestis KIM delta pgm, which is essentially fully virulent from an intravenous route, so our inspection is going to be intravenous, and it will model systemic plague. We have a very potent rabbit polyclonal antibody antiserum against LcrV that we give to the mice in one dose the day before we infect, and we give a high dose of bacteria to allow us to follow the dynamics of early protection by viable numbers.

So, in our control mice, which are C57 Black 6 mice, we look at the gold and the blue symbols, if the mice are given the anti-V shots and they control bacterial numbers, these mice will live. If they were given instead a nonprotective anti-Yop and antibody, then, they experience a runaway infection and will die starting around day 4.

So, the first question we wanted to ask

was can you get protection in the absence of V's effect on IL-10?

We can address this in two ways. On the left we use IL-10 knockout mice, and these are highly polarized toward Th1 responses and are actually remarkably resistant to plague lethality, but nonetheless, you can give a high enough dose and kill them, so we tested, and the answer was yes, we got exactly the same dynamics, these mice are protectable by the antibody, and we did it a different way over here.

I am showing just one time point where we ablated IL-10 with a neutralizing antibody against it, and the controls with anti-YopM received neutralizing anti-IL-4.

So, the answer is yes, you can protect using anti-V in the absence of effects relating to IL-10 production from V, and so the issue was then, what is it. One thing that we followed then is the effect on Yops delivery, and we have confirmed in a number of assays the findings of the Welkos group and others that anti-V does partially prevent the

delivery of Yops to adjacent cells.

In this context, it is useful to look at this particular assay, which is a phagocytosis assay using a double fluorescence method. So, if the infection is done in the presence of the nonprotective antibody, the anti-YopM, then, most of the bacteria are extracellular and stain red, and if it is done in the presence of the protective anti-V antibody, then, most of the bacteria are intracellular.

So, that raised a question, does the bacterial location affect the expression and delivery of Yop?

There was an experiment that Roland Rosqvist and Hans Noskos did back in 1990 with ED76 in HeLa cells that indicated that the only bacteria producing Yops were the extracellular ones, and we wanted to know is this the case for Yersinia pestis KIM and J774 cells.

So, here is the design. We are going to make a delivery of YopH into the cytocellular fraction of J774 cells after four hours of

infection. We have set it up this way. We have a whole bunch of cultures. Some of them are infected in the presence of the anti-V antibody, some with anti-YopM, some with no antibody, and this goes for 30 minutes to allow phagocytosis to take place.

There will also be an initial burst of Yops delivery during this time, and then triplicate cultures are divided as follows. So, one gets a dose of gentamicin which is sufficient to inhibit protein synthesis by extracelluar Yersiniae, so that now the only further Yops that are going to be delivered would be by intracellular bacteria.

One gets a mixture of antibiotics that will kill all Yersiniae inside and outside in 15 minutes, so they will be essentially no further Yops delivery, and one gets no addition. So, here, we are going to get Yops from both extracellular and intracellular, and this goes for an hour. That is washed away, and the antibody treatment is restored, and then the incubation is finished.

So, here is what we got. The no antibody, anti- YopM, anti-V, and the three drug treatments

for each, this is noninfected. I would like you to focus on the comparison of gentamicin and mix in each case.

The mix kills everything. You get no further Yops. Gentamicin, you get delivery from intracellular bacteria. They are pretty much the same. There is no delivery from these intracellular Yersiniae, and what is happening here is what happened during that first half an hour.

If there was no drug treatment, then, you got tons of Yops still delivered, presumably by the extracellular bacteria in these control treatments. With anti-V, when the bacteria are mostly intracellular, you obviously get less Yops delivered.

So, that raises the question: Is antibody inhibiting Yops delivery and causing phagocytosis, or is antibody causing phagocytosis and subsequently inhibiting Yops delivery?

So, to test that, we asked can antibody be effective in preventing delivery of Yops to cells that cannot phagocytose.

So, we did these infections in the presence of various concentrations of cytochalasin D, and when there was no cytochalasin D, then, yes, you do get some inhibition of Yops delivery by anti-V antibody compared to the controls, but as we increase the cytochalasin D, we got progressively less and less effect of the antibody.

So, antibody is not effective against bacteria, against cells that cannot phagocytose, and that leads to the question of what is it about antibody, is the ST portion of the antibody important for its efficacy, and the answer is yes.

This is actually in full agreement with an experiment published by the Welkos group. So, with no antibody, most of the bacteria are extracellular. With full length V antibody, you get promotion of phagocytosis, but if you make FABs, it doesn't work very well.

So, this is what we think is happening, that antibody is actually promoting phagocytosis and consequently, you get inhibition of Yops delivery rather than the other way around.

So, if phagocytosis is so important, and we think it is important to protection, then, what are the cells that are important or mediating this. So, we evaluated the relative contributions of macrophages and PMNs.

To do macrophages, we took advantage of a recently available transgenic mouse model that allows you to conditionally ablate cells of the macrophage lineage, and so we are seeing here then our usual control mice of the nonablated that receive anti-V or anti-YopM, but the ablated mice that receive the anti-V, it really made no difference in spleen.

So, the macrophages are either redundant or not necessary for protection in spleen. In liver, we got a small effect, maybe about 10-fold less ability to contain bacterial numbers, so macrophages do make a contribution in liver.

We ablated PMNs with an antibody against ra-1, and in that case, it made a huge difference in both organs. You get total loss of ability to contain bacterial numbers.

So, I have shown you that, as we all know, and I really believe, anti-V promotes phagocytosis and that this is actually crucial for its protective effect; that Yops are not delivered by phagocytosed Yersinia pestis. Yops are crucial for growth in organs. I didn't show you those data, but they are, and that is what anti-V is doing. It is preventing growth and thereby you never get the bacterial numbers to produce enough V to even have a big effect.

I told you something about mediators of early protection, that PMNs are really important, and macrophages also make a contribution.

This work was almost entirely done by a postdoc Sasha Philipovsky, some help from Clarissa Cowan, an advanced technician, and another technician, Michael Gray.

I will be happy to answer any questions.

[Applause.]

DR. : Very nice, Sue. This is just a comment. You think it goes to say that an earlier study by Friedlander and colleagues showed

that the immunization of mice with another protein which is thought to aid in the translocation process, namely, YopD, doesn't even nearly promote the same protective efficacy as antibodies against V.

That will corroborate the notion that what the V antibodies may be doing is not blocking the injection of YopD.

DR. STRALEY: We haven't evaluated what antibody against D does, YopD does. I really can't address that relative efficacy.

DR. : What the data show is that the antibodies against D do not necessarily block the injection of Yops from extracellular bacteria.

DR. STRALEY: If the bacteria remain extracellular, they deliver Yops, I think is the fair way to say it, but somehow antibody against V is also promoting phagocytosis, and I think that that is what is causing the downregulation of Yops that we see.

DR. : I am curious if you are not far enough to find out if acidification of the

vesicle is necessary to prevent infection, because it would seem that infection could conceivably occur through the vesicle itself.

DR. STRALEY: We have not tested that.

DR. : Do you think that the effect of anti-V is specific to the FC receptor in that it is that mechanism of phagocytosis that is critical, or do you think if you could promote rapid phagocytosis by any mechanism, you would get the same response?

DR. STRALEY: Well, let's see. I guess I can draw on the published literature first, that the Welkos group did show, and Sue may have shown this slide, that you just use an anti-Yersinia antibody, it can protect.

I am thinking of promoting phagocytosis. I guess she didn't show the phagocytosis data today. We don't really know the answer to that at this point. We did test whether we could protect in FC-gamma 3 knockout mice, and you can, but mouse PMNs don't have FC-gamma 3, so I am not sure it was a good test. We are actually doing that right now

to try to address that very question.

DR. : I guess I am wondering also if it's possible that the antibody, if it's binding to the tip of the type III secretion system, it could be essentially preventing the type III secretion system from contacting the host cell at the same time that it's promoting uptakes.

Do your experiments address that possibility?

DR. STRALEY: Well, I guess in the experiment where the cells could not phagocytose, we had lots of antibody around, and we got tons of Yops delivered, so the antibody is not preventing delivery directly.

We have done one experiment also with FAB primes in vivo to see if it would protect, and the answer was no. We are repeating that now. I don't know if anybody else has done that experiment.

DR. : One other question. Do you know the protective epitopes of this antibody preparation you are using, have you mapped what regions of D are recognized by this antibody?

DR. STRALEY: I imagine it's the whole protein. Interestingly, FABs of this antibody will reduce the IL-10 effect, so I am suspecting that a range of epitopes are represented.

DR. LINDLER: Leah Scott from DSTL is going to speak about the marmoset as an immunological model for plague, just one added five-minute talk.

The next speaker is Dr. Stephen Smiley from the Trudeau Institute to speak about cell-mediated protection against Yersinia infection.

Cell-mediated Protection Against Yersinia Infection

Dr. Stephen Smiley

DR. SMILEY: Thank you. I thank the organizers for inviting me. I am a relative newcomer to this field and I am looking forward to speaking to you today.

So, basically, we are asking this question, can vaccine-primed CD4 T cells protect against pneumonic plague, and I am not going to answer that question today, but I am going to show you the tools that we are developing that we think

will let us address that question.

We have already heard today that Yersinia can be intracellular bacteria and that it has been established that interferon-gamma and TNF-alpha, which are products of cellular immunity, can protect against Yersinia infection, and it turns out that it is well established that CD4 T cells are important players in both cellular and humoral immunity, but the actual functional roles during Y. pestis vaccines, I don't think it has been evaluated decisively. It has been shown that they can be stimulated but not shown that they can protect.

So, V protein has already been discussed today in some detail, as has the fact that the vaccine that is under development by USAMRIID of the F1-V fusion protein fails to fully protect primates. What I was told about that was that in some animals, there was a late breakthrough of disease, and what that suggested to me is one possibility is that perhaps there are reservoirs of bacteria that antibodies were unable to clear and

that subsequently, it led to disease.

So, obviously, that is just a hypothesis and so is this, which is that appropriately primed CD4 T cells may be able to direct cellular immunity at those intracellular reservoirs, thereby improving plague vaccine efficacy.

I just want to stress at the outset, though, that our intention is, our belief is that these cell-mediated protection will synergize with the humoral immunity. I am not suggesting that we replace antibodies.

Our approach to this is relatively simple. At the outset, we are going to define CD4 T cell epitopes in V protein. That will give us tools, a way to specifically prime CD4 T cells. Our plan was to assess the protective capacity of those cells, as I said, to assess whether a cellular and humoral immunity can synergize in combating plague.

This is just a schematic of V protein, and these are the peptides that we had synthesized in 96 well format that spanned V protein, and we then screened these peptides in an ex vivo assay.

To do that, we got the F1-V fusion protein from Jeff Adamovicz, from USAMRIID, and we vaccinated E6 mice with that protein to get a strong Th1 type response. We then purified those CD4 cells from those mice after six days, and in vitro did a re-stimulation assay looking at responses to these peptides.

What we were able to clearly see as we scan across this slide, there are 63 individual peptides that were screened. In the top are the CD4 cells from the F1-V primed mice, and in the lower panel are CD4 cells isolated from OVA-albumin-primed mice as control, and you can see that there were regions of V peptides, V1, V2, and V3, where we saw strong responses.

On the far right you can see the controls, the OVA responded to OVA only, and the F1-V in culture only revoked a response from F1-V primed cells.

We went on to further define these epitopes by making a second set of peptides in which we truncated at either the amino or carboxy

terminus by 2, 4, 6 amino acids, and then rescreened in the same type of assay, and that allowed us to find map with specific epitopes.

Those are shown here on this slide, which compares the V proteins from Y. pestis, enterocolitica, and pseudotuberculosis. The peptides that we use is V1, V2, and V3 for vaccination studies on bold, and the boxed are the better defined epitopes by that second series of studies.

You can see that they are completely conserved among Y. pestis, enterocolitica, and pseudotuberculosis. I suspect that it is just coincidence, but it is a useful coincidence for people studying those infections, as well.

It is also useful to us, and I will show you that in a second.

If these are true CD4 epitopes, then, one should be able to vaccinate mice with these small peptides V1, V2, and V3, and elicit responses to those peptides, and that is what this slide shows.

Here, we have vaccinated either with a

control negative peptide V neg, V1, V1, or V3, again in CFA, and then in this particular experiment, we vaccinated on day zero. We then vaccinated with the same peptides in IFA at day 30, and this is looking at a recall response in vitro. It's an Ellis spot response where you can measure the number of interferon-gamma producing cells in that culture.

You can see that the V1 evoked a response from V1 in culture, V2 from V2, and V3 from V3, so these epitopes prime antigen specific CD4 T cell responses.

We then wanted to ask whether these could protect, and in the enterocolitica system it has been shown that cellular immunity by CD4 T cells can be protective, and also enterocolitica is an agent that we could work with easily at that time, so we went there.

Here, I am showing protection. There was significant protection. There are the exact same animals that I just showed, from the same cohort of animals that I just showed you that Ellis spot

data. You can see that the mice that were vaccinated with V2 showed significant prolongation of survival upon infection with 10^4 of enterocolitica IP.

So, our plan, of course, is to go on and test this with Y. pestis. We set up a collaboration with David Perlin at PHRI who is setting up the intranasal model, but before that got underway, the pgm-minus strains were released from the select agent list, and we were able to test those ourselves, so we got one KIM D27 from Robert Brubaker, and in the exact same type of scenario I just showed you on the previous slide, we were unable to see protection using that strain.

We have tried that several times, and so far that type of protocol has failed to protect against IP KIM D27. I know IP is not the preferred route that people are studying, and we are looking at other routes at the present time.

I just wanted to point out I think as some people have today, that these pgm-minus strains are attenuated, but they are conditionally attenuated,

so by the IP route, this is just an LD50 curve, and you can see that the LD50 is quite low, so they are not so attenuated by the IP route, likewise by the IM route, they are not particularly attenuated. So, we plan to use this for our future vaccination studies.

So, what we are now focusing on is whether CD4 T cells can synergize with humoral immunity to protect. As I said, we didn't expect them necessarily to protect on their own.

In the types of studies that have classically been done along these lines, one can transfer and give serotherapy the day before doing an infection, and then give the infection the next day and measure the capacity of that serum to protect.

But in our types of studies, that wouldn't be appropriate because in these types of studies, what happens is the serum unblocks infection, and we want to ask whether T cells can contribute to clearing intracellular bacteria, so we need to have cells infected first.

So, we decided to try this other protocol where we would first infect and ask whether serum can protect the next day, after the infection is in place.

The answer is--I am not sure whether this has been done or not in the past--but it clearly can. That is the block there, the round circles. That is serum given day plus 1, again with this 10^4 IP KIM D27 model.

So, post-exposure serum therapy can protect, and it is actually extremely potent. We have been quite impressed. As little as 3 microliters of this serum protects. I forgot to tell you what this serum is, I apologize. When we do a sub-lethal KIM D27 infection, so that is 10^2 IP, we can then collect the serum from those animals out 30 days, and that is the serum that we are using in these serotherapy experiments.

So, that serum is extremely effective, and it has allowed us to do the last experiment that I want to show you, and that is to develop an assay for protective cellular immunity.

So, here you will see I have switched to MUMT mice. MUMT mice lack V cells, they can't make antibody responses. They are genetically deficient in D cell production. So, what we have found we can do in these mice is we can give them the KIM D27 IP infection, and then the next day give them this serotherapy, and if one does this that, these mice survive.

What we wanted to know was did that process vaccinate them with cellular immunity that could protect against a subsequent day 50 challenge. An appropriate control was to use mice that got a sham infection initially, got the serotherapy, so if there is any leftover serum from the lower infection, it should still be there from the upper mock control, as well, and then challenge those mice.

We need to repeat this, but in the first experiment, it was really quite striking. It appeared that all of the mice that were vaccinated, all the MUMT mice which can't make antibodies were vaccinated in a way that allowed them to survive,

were able to then survive a secondary infection with 10^4 IP KIM D27.

So, we are quite excited about this. I think it is pretty clear evidence that cellular immunity can protect against IP plague, and we are moving on to look at the other models.

In closing, I just wanted to bring out a point that I don't think has been discussed much, but for cellular immunity, I think we need to rethink what are the right targets.

V protein is clearly a good target for humoral immunity, but for cellular immunity, in order for cells to attack infected cells, we need the Yersinia proteins to be expressed within those infected cells, and I am not sure V protein is the right target in that context.

We have got assays that we are setting up to try to identify what are the right targets in vivo, and I would be happy to talk to people about that, but I don't think I have time right now.

So, in closing, I just want to thank Michelle Parent and Kiera Berggren who have done

most of this work, my Trudeau Institute colleagues, Jeff Adamovicz for providing the F1-V and support, and Bob Brubaker for providing the KIM D27.

Thank you.

[Applause.]

DR. : Very interesting presentation. I just wondered whether you thought that the T cells that you have identified in the C57 Black 6 mice might be conserved in other haplotypes of mice or not.

DR. SMILEY: I suspect that they won't be. I think that is one of the difficulties of looking at cellular immunity, that since it is all MHC restricted, it will depend on which strain you are looking at. I hope others are looking at other strains.

DR. LINDLER: Any other questions?

Thank you.

The next speaker is Shan Lu from the University of Massachusetts. I think he is going to speak about search for an optimization of protective antigens for plague vaccine development.

Search and Optimization of Protective Antigens for Plague Vaccine Development

Dr. Shan Lu

DR. LU: First, I would like to thank the organizers for inviting us to present the data here. Also, I think that this is really a well-organized conference. Being a beginner to this new plague vaccine field, I thoroughly enjoyed the informed conference here today.

When we start plague vaccine, actually it was started as a graduate student vacation project. Being someone working in the vaccine field for 10 years including some of the HIV project, I hope people appreciate how much you have here. Actually, you have an animal model, you have something of the immune correlates, and actually you know what antigen they protect.

So, when we started, we look from different perspective, that is, what is the issue we want to address here, how can we improve rather than reinventing the whole wheel. So, I thought we should divide it into two parts.

One is how can we improve the immunogenicity part and then the second one, especially in the current regulatory and society environment, how can we improve the safety of the future generation of plague vaccine.

In the immunogenicity part, we know that at least the two protective antigen has been identified, however, the quality, especially the production, how do you put the two antigen together has been some issue. When you fuse them, do they really form a big aggregate or a functional antigen?

Also, we realize including today's presentation, there is a chance we can identify new or novel protective antigens. The other thing is how can we deliver antigen. We know the live attenuated approach probably is not viable nowadays, so what are the other choices for us, especially in light of induction of several immune response, like presented by Steve right before my talk, and also, of course, today we talk about what is an acceptable animal model because many

modalities relate to what model you are looking at.

The safety part I thought is also very important, because that relates to how do you protect, produce? Produce vaccine, it is not what type, live attenuated or not, it is really standard what you can really have a well-defined product to go into human trials rather than you know the antigen.

Also, how do we select additional antigen? And then finally, how do we administer into potential human population.

So, our strategy at U. Mass was based on the following premises. One is built on our previous experience on design of novel vaccines. Our focus was not on the modality of what type of vaccine, rather identify immunogenic antigens, because a vaccine is the business of antigen. We need to pay more attention on that.

The second part, is how do we deliver, what is the technical approach. We focus on the subunit based approach, but subunits in my laboratory expanded to include both the recombinant

protein, as well as DNA as the subunit.

Actually, DNA, to me, actually is the best approach to develop subunit vaccine. You can bypass many technical difficulty when you deal with a protein antigen.

Then, finally, we further tried to use efficient system to screen for new protective antigens.

So, let's show you the first, just an example, this study. Actually, most of that already been published in recent issue of Vaccine, but I just want to give you more detail here.

So, in this first study, we included three potential antigens: V and F1, that is well known as a potential protective antigen; we included Pla, Jon Goguen has been one of the pioneers showing the pathogenesis of Pla, so we also want to see whether this can function as a new protective antigen.

As you may read in literature, our colleagues at UK have done work in vaccine, especially use the B antigen. They have used a gene gun, which is my favorite approach. They also

optimize, they also use the DNA protein. Those are all the approaches I like, however, they still did not see -- they reached some level close to recombinant protein.

So, one trick they have not done, which I do a lot in my lab, is look at the leader sequence here showing as a black box. The difference between bacteria antigen and the DNA vaccines is the DNA vaccine has to be expressed in the main system, and for the V antigen, they don't have a putative leader sequence.

But we know that, when you have an antigen, a special post-secreter, antibody antigen, you need a secretion. So, that is why I am making some kind of F1 antigen, is very immunogenic because it secretes.

Also, Pla has a hydrophobia reason. We don't know whether that is a leader or not, but still it is a popular strategy or at a leader sequence. Actually, we find a very different type of a response.

So, here is the immunization schedule,

very simple. Every 4 weeks or monthly, we give a DNA immunization. We use a gene gun. Each animal, we give 6 micrograms of DNA. I just want to emphasize this is not an optimizing. You can see the protective efficacy of our approach.

Then, we wait a long time. That is not because we designed it that way, because our 303 lab was not available, we have [inaudible], and so on and so forth. Finally, we have a boost and challenge, and later I can show you, this actually not relevant, we can shrink that, earlier challenge.

Here is the RB antibodies. I get a response by ELISA. You can see the V works very well. F also works. The tPA maybe improve a little, but not too much from the binding antibody, but the Pla was completely negative. That shows you Pla is not immunogenic by this design.

Then, I will just go quickly to the key part, so you can see that for the tPA of wild type, the tPA actually have more secrete--they have early rise. After one immunization, you see very tight

antibody response, but after immunization 2 actually, they reach very similar level of a response, so the binding antibody, tPA-V or wild type of V, they are very similar.

However, the interesting thing is here, better protection. This is the first time you study, we use a 5,000 CFU, which is equivalent, about 100 LD50. You can see that we see three patterns, three antigens. This is the V group here, this is the F1 group, this is the Pla.

Pla has no protection at all, the same as the control. We see in 3 to 5 days all animals without exception all die using the strain 100. That is the one Jon Goguen used for many years.

You can see the F1. Whether you use the tPA or not, they are in similar range of partial protection, and then the real interesting part is the V antigen, very clear cut. With the tPA, no exception, all protected. You can follow that with 2 weeks or even longer. The wild type, as we expect from V antigen, there was protection, but a partial, the same as the F level.

So, this confirmed previous work that V can protect. So, the next question we want to ask, can we give a higher challenge dose. You can see here, this is the same 5,000 CFU, this is the 20,000, and this is 80,000, which is about 300 LD50. This is all intranasal challenge, by the way, and under 50 microliter, I agree with a comment earlier, what we do intranasally is we anesthetize the animals, you draw up the Y. Initially, Jon was a little bit suspecting whether that would work, but if you see it once, you know that they will draw up everything, very reliable technique.

So, here you can see that after the challenge dose, 90 percent protected with tPA. The wild type at the baseline is about 20, and then when we go higher dose, 20,000 CFU, you can see about a 80 percent, then, 70 percent.

If we put all the data together, just compare here the control group, all the animals now survive, and also I want to comment here, the size of animal group were increased to 10 animals, so 90

percent for--for the tPA-V at 500,000, and 80 percent, that's 20, and 70 percent or 80 percent.

Unfortunately when we transfer the 5, this number lost. Here, at the two dose, all significant. If we combine all 3 together, you can see the p-value is very, very significant, so clearly the wild type and tPA-V are very different qualitatively.

So, the question is what happened here, they are all V antigen. They should be the same V antigen. So, it is very interesting. We want to prove our antibody, then, we find something very interesting, that is, here, with the tPA, they form the dimer or tetramer. For the wild type, you don't see that, and they have less secreted.

Because of time, the reason I am not showing the other--if you look at our paper, you will find if we over-express or produce here, this is wild type, you can still see just a band, don't form oligomers.

The binding is very strong for V antigen, so that is why when you make a fusion protein,

sometimes [inaudible], you will see that, you will reduce it back to single band. Prove that.

The next question is interesting. So, we look at what are the subtypes as a simple way to measure Th1, Th2, as we discussed earlier this morning. You can see that because we use gene gun, so we can see a predominant IgG1, a pH of 2, as we expected from a protected antibody.

However, the simple fraction of the IgG2A, which is representing Th1, actually was increased with tPA type rather than the wild type, so suggesting somehow when we change the leader sequence, the 40 of the protein is different, as we see from the previous western gel. So, there is some confirmation difference.

We know that the leader sequence is actually very critical after translocation, protein differences. We know the so-called immune suppressed function, probably is the N-terminus. So, whether that 40 actually affect that, we don't know. So, that is a very interesting question.

Given the time, I will just quickly show

you some other data very quickly, just one minute.

So, we also look and use the same technology to quickly screen like we are doing here, we look at YopD, YopB, and YopO, because you can see, sometimes a bacteria antigen has two hydrophobic domains or just has one, so we made all kind of antigen engineer, remove them or not removing, add a leader or not, then, you can see the result here.

So, with YopO, the wild type, they were mainly intracellular, but we make a tPA and allow to secrete. With YopB, unfortunately, again, Microsoft shifted here, so you can see here, we have N-terminal here, very well expressed, we can selectively express certain domain, [inaudible] or you have the tPA with no change at all. You can see if you remove the hydrophobia region, you will actually induce a secretion, improve the immunuogenicity, and the same thing here with YopD, so we are looking at whether this antigen can provide any protection. I can tell you briefly, basically, we did not see a major protection.

However, what I want to show here with this technology, you can quickly screen many antigens in a reliable, protecting model without going through very complicated protein production process.

So, this is our strategy for the future study. We believe the DNA or DNA-plus protein is a very viable approach to generate emerging vaccines as most people agree in this audience, and we want to have proof of efficacy, protective antibodies, plus proof of cell-mediated immunity. Whether that is CD-4 or even CD-8, we don't know yet, and also we use DNA as a protein, as a technical protein.

We believe that this is a safe and very easy to administer, and the most important thing, if we use subunit and DNA in the future, the plague vaccine can be mixed with other biodefense--so the soldiers, when the go to field, they don't have to receive 20 needle sticks. They can use probably one or two.

So, I will stop here. Finally, I want to thank my collaborators. At the top are the people

from my laboratory. I want to thank my colleague, Jon Goguen, who gave all the guidance and without him I don't think we would make such a program.

Thank you. I will stop here.

[Applause.]

DR. LINDLER: Any questions?

Thank you.

The next speaker is Kathleen McDonough from the Wadsworth Center speaking about profiling differential gene expression in Yersinia pestis as a tool for vaccine target identification.

Profiling Differential Gene Expression in Yersinia Pestis as a Tool for Vaccine Target Identification

Dr. Kathleen McDonough

DR. McDONOUGH: I want to start by thanking the organizers for the invitation to speak today about a project that is very new in the lab. Unlike the plague doctors of the Middle Ages, we certainly have a tremendous number of tools, particularly most recently the availability of complete genome sequences, but I think although we are rich in these technologies, we are also, as we

have seen today, still fairly poor in answers about what makes plague bacilli really tick.

So, what we are interested in is identifying some environmentally regulated plague-specific gene products that may also be useful for pathogenesis for the organism and, from our perspective, for diagnostics in vaccine design.

Classically, in terms of the Yersinia, DNA relatedness has been not a good indicator of biological similarity or at least in terms of pathogenesis, and so our approach has been to think about looking instead at expression profiling to get at some of the more unique pestis attributes, and, in particular, as we have been hearing all today, the disease that pestis causes is certainly very different than the disease of either the other enteric pathogens, the enterocolitica or pseudotuberculosis, and, of course, only Yersinia pestis is transmitted by fleas.

So, in terms of thinking about expression profiling, the most immediate choice we had to make was protein versus RNA, and we have chosen the

proteome approach for a couple of reasons listed here.

In particular, protein is a more final product than RNA, and very importantly, we think that this then allows us to get to posttranscriptional regulatory products that we think may be important particularly for plague.

Protein also has more direct potential than RNA as a direct vaccine target, and, of course, we have some additional advantages to doing proteins over RNA in that we can fractionate our samples, and so on, before we look at them if we want to get, in particular, for vaccine type of development, secreted or membrane-bound antigens.

So, back onto the idea of posttranscriptional regulation and thinking that it may be something of particular importance for Yersinia pestis. A recent paper out of Bob Perry/Jackie Featherston's lab, the HMS phenotype that is critical for blockage of fleas and therefore transmission by the natural plague rodent/flea route is posttranscriptionly regulated,

and an older paper out of Brubaker's lab, it is shown that some of the Yop routines are degraded by the plasminogen activator on the pestis and plasmid at least in vitro, and whether this happens or is important in vivo or not has not been followed up.

I am going to skip the sort of technique slide there. I think most folks are pretty familiar with 2D GEMS or 2D GEL electrophoresis in mass spectrometry, and just move on to some of the applications.

This is actually a study from a different project in the lab on TB that illustrates the point of how 2D GEMS can be particularly useful for identifying posttranscriptionally modified bacteria, and this is just looking at differential protein expression in a vaccine strain of TB, and the only important things to get here are that with some of these identifications that are shown here, the two most prominent differentially expressed one, this Number 7, the PE PGRS6, and the GRO-EL2 are both actually posttranscriptionally modified, as well as transcriptionally differentially

expressed.

Those are the two that are shown here in blue, and you will notice that at the protein level, GRO-EL2 is differentially expressed or induced 10-fold in the one condition over the others, but only 2.3-fold at the RNA level. Likewise, 27-fold deduction at the protein level for this PE PGRS protein, and only a very minor or relatively minor increase at the RNA level.

So, the 2D-GEMS is particularly useful for looking at the total protein effect. I should say that of those two prior proteins, one of them is a protease cleavage event, and the other is a lot more likely a translational regulation.

So, the other thing that we would also get that wasn't done, if there is any kind of other protein modifications, methylations or phosphorylations, et cetera, they will ship them in gel, and they will come up in this kind of analysis.

So, in thinking about what kind of regulatory conditions would be interesting to look

at, a first temperature in calcium or the other really classical regulators for plague, as well as the other Yersinia.

Iron, as well, a tremendous amount of good work has been done on this, particularly out of Bob Perry's lab, but the rest of environmental conditions have not been well addressed in Yersinia, and in all of them, or each of them may also have a role in mammalian host particularly with respect to the time they may spend intracellularly within macrophages as has been alluded to several times today.

So, the data that I wanted to just show you today has to do with looking at hypoxia, and one of the reasons that we chose hypoxia is because in other organisms, hypoxia has been a very good signal to look at to identify genes that may be induced or regulated within the macrophage.

So, in particular, the thing to key in on here is that in terms of oxygen in atmospheric air, it is very high levels. Also, in the lumen of the lungs, the oxygen is also going to be very high,

but then once you are inside a cell, the oxygen will drop quite a bit, down to about 2.6 percent.

Another thing that happens in the mammalian host that is different from what is in the environment is that the CO_2 levels in the environment are very low, but almost anywhere you go, in a cell or out of a cell, within a host, is also CO_2, so in our hypoxia conditions, when we modulate the oxygen, we also include CO_2 when we are thinking about mammalian conditions.

So, what this has shown here is just a little profile of some proteomes. On top is Yersinia pestis, and on the bottom is pseudotuberculosis, and either on the left, ambient air conditions or hypoxic conditions, which were 1.3 percent oxygen and 5 percent CO_2, and the things to clue in on here, the real question we were asking, are there differences between pestis and pseudoTB in this condition.

So, the boxes are showing proteins that differ between pestis and pseudotuberculosis. So, for example, here you have got that little doublet.

You have got the top guy here. He is missing here, he is missing here, and so on.

Also, the circles are going back and forth and showing intraspecies differences in pestis or in pseudoTB, and there is others that are not marked here that are lost in the translation.

But the other things to keep in mind is that we use strains to try to match, mostly for the chromosome, and so that we wouldn't have as much interference in terms of differences from some of the extra plasmids, and so both pestis and pseudoTB were both LCR-minus.

The pseudoTB isolate, we used is a serotype I that has the high pathogenicity, and also the pestis that we use is 10-plus, so it also is missing the pestis in plasmid.

In terms of extra DNA that we know is there, the PMT1 plasmid is present in pestis, and not pseudoTB. So, what we will move on with is also looking, as well, at the different contributions of each of the different plasmids in terms of regulating chromosomal genes.

This just shows a later time point. The time point I just showed you was one hour in hypoxia. This is now 24 hours in hypoxic conditions, and this is just pestis, and this is showing a number of differences between the ambient protein expression versus the hypoxic protein expression.

So, I have shown you so far, or what I have shown you, all I am going to show you today, is essentially the 2D-gel electrophoresis. We think it is a useful approach for identifying pestis-specific responses to the environment.

The pestis proteome does change in response to the hypoxia, and pestis and pseudoTB also respond differently to these conditions. Where we are going in the future and currently is that we will analyze the response of pestis to see the additional environmental conditions that I mentioned. People have ideas about other things or in terms of prioritizing things.

We are certainly interested in hearing them. In addition, what I didn't show you is any

metabolic labeling differences, it was all just Steady State approaching comparisons. With metabolic labeling, of course, we see lots more changes although they are harder to follow up in terms of the mass spec. protein IDs.

In addition to doing the 2D-GEMS, another mass spec. approach is ICAT technology or the isotopically coated affinity tags. We are doing some of that, as well. It essentially bypasses the 2D-GELS, and it is a very complementary approach to the 2D-GELS because you are able to analyze kind of different sets of proteins, as well as some overlap.

Then, of course, for the future, we will move on with the proteins that are identified and characterize them with respect to regulation and function, and their potential as vaccine or therapeutic targets.

The people simply I mention here would be Michael Gazdik had done the TB-related gels that I showed you, and David Schaak did the plated gels.

Thank you for your attention.

[Applause.]

DR. PERRY: Bob Perry, University of Kentucky.

Kathleen, is there a difference in the growth rate between your atmospheric and your hypoxic strains? Does it really drastically change the generation times or are they growing about the same rate?

DR. McDONOUGH: We haven't analyzed that really carefully yet, so I can't say total, but there was nothing really dramatically obvious in terms of culture densities, but this is the kind of thing we used to go do.

DR. PERRY: I just sometimes think we need to sort of monitor that and then see if some of the changes are not due to oxygen, temperature differences, but growth rate differences, and do that by adjusting your growth rate with other deficiencies.

I think there have been some microarray studies where they haven't taken that into account, and you see a whole bunch of weird genes that are

iron regulated, but I am wondering if they are growth rate regulated instead.

DR. McDONOUGH: Nothing looked obviously different. They didn't seem really challenged. The other thing is they were only in for an hour.

DR. LINDLER: Have you been able to map those to specific regions in plague or pseudoTB, where those gene products are coming from?

DR. McDONOUGH: Not yet. Next on the list. One of the things we typically do. You get a lot of variability in terms of gel-to-gel is typically very consistent, but in terms of biological repeats, and we have learned from experience that before we go on and identify things by mass spec., we end up setting up really rigid criteria, so that we like to have at least three biological repeats of proteins that are reproducibly changed before we move on and do the I.V.'s. So, that is still in progress.

DR. LINDLER: Thank you.

The last speaker is Leah Scott from Defence Science and Technology Laboratory. She is

going to speak about the marmoset as an immunological model for plague.

The Marmoset as an Immunological Model for Plague

Ms. Leah Scott

MS. SCOTT: Good afternoon, everybody. Thank you very much for your forbearance at the end of a long but productive day.

I would just like to spend a few minutes highlighting some issues that I think are terribly important to us all. Particularly, we have heard from Louise about the importance of nonhuman primate models in this area, and this is an option that I just want to raise with you. We will be around for the rest of this evening and tomorrow if you want to discuss things in greater detail.

For those of you who may not be familiar, familiar with the marmoset, here they are - small, new primates 350 to 450 grams. I will just say very quickly, this is background, what we know about immunologically, I will allude to work in progress, and finish off with some remarks about other sources of information.

We know, worldwide, the common marmoset is becoming much more popular and has been widely used in many areas of research including, as it says there, including a number of fundamental applied research areas in regulatory studies in diverse areas, particularly in neuropharmacology, behavior and toxicology. We know about those issues. They have been around for a long time.

But specifically in the context of the world which many of us in this room live, in the UK, the marmoset has been extensively used to elicit the effect of nerve agent poisoning, and it continues to be absolutely pivotal to us in bridging guinea pig studies to human studies when we are talking about the development of nerve agent pretreatment and therapy.

What our plans are for the future, we are looking at marmosets. We haven't done plague in these animals yet, but we have plans to do so in the not too distant future. We aim to characterize the model, understand its relative strengths and weaknesses, which is a fundamental approach that we

have right across our work here, and we aim to do that, as I say, in the next six months onwards.

We have been involved in characterizing the marmoset as a model in immunological studies because of the middle bullet there. The marmoset has been used in our laboratory in a very high profile study over the last four years to look at the effects of multiple vaccinations in the context of Gulf health.

Previously, it had not been particularly well characterized as a model in such studies, and we had to build upon one or two case studies and build up the toolset, so that we can understand the impact of vaccination in this model. We are now in a position to do that.

The big issue, of course, with the marmoset is because of its small size, and its incredible productivity in terms of laboratory management and captive breeding, marmosets tend to have twins or triplets twice a year, and it can be used from age 11 months to 12 months onwards.

So, those of you familiar with large

primate studies will see that there are enormous benefits to be gained from that. Moreover, their captive management is relatively easy, and that includes in high levels of biocontainment. So, certainly worth considering in this context.

This is just a summary of what we can do at the moment. In view of the lateness of the hour and the short time that I promised to talk for, I shan't go through it all.

Suffice as to say we have the toolset. I have some exemplar data. Come and see me afterwards or tomorrow, and we can discuss those issues. Just wanted to flag up the big issue.

Many of you in this room, all of you in this room will know our existing plague team, but these are a few other folks, some of my other colleagues at DSTL, what I would call the Parent Marmoset Immunology Team, who have been looking at marmoset vaccine studies in the context of Gulf health.

Gareth and his team would be very pleased to help. I would also like to draw your attention

to the European Marmoset Research Group, which was founded more than 10 years ago now, and is developing as a very strong information base, the discussion of such issues, and more recently, the development of the Marmoset Research Group of the Americas. The web site address is there.

Thank you very much for your forbearance and will look forward to talking to you.

[Applause.]

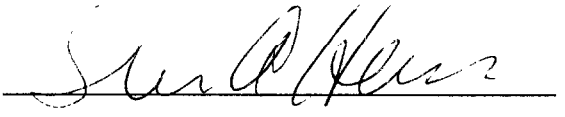
DR. LINDLER: Any questions?

I would like to thank the organizers and I will turn it back over to them. Thank you.

[Whereupon, at 4:48 p.m., the proceedings were recessed, to reconvene on Thursday, October 14, 2004.]

CERTIFICATE

I, **SUSAN A. HARRIS,** the Official Court Reporter for Miller Reporting Company, Inc., hereby certify that I recorded the foregoing proceedings; that the proceedings have been reduced to typewriting by me, or under my direction and that the foregoing transcript is a correct and accurate record of the proceedings to the best of my knowledge, ability and belief.



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